



2013

Characterization of Inducible Regulatory T Cells: An Umbilical Cord Blood Model

Rebecca Anne Krier
Loyola University Chicago

Recommended Citation

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LOYOLA UNIVERSITY CHICAGO

CHARACTERIZATION OF INDUCIBLE REGULATORY T CELLS: AN
UMBILICAL CORD BLOOD MODEL

A THESIS SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
MASTER OF SCIENCE

PROGRAM IN INFECTIOUS DISEASE AND IMMUNOLOGY

BY

REBECCA ANNE KRIER

CHICAGO, IL

MAY 2013

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ACKNOWLEDGEMENTS

First and foremost, I would like to thank my mentor, Dr. Makio Iwashima for providing me with the opportunity and guidance to better myself as a scientist. This thesis would not have been possible without his encouragement. I would also like to thank my dissertation committee, Dr. Christopher Wiethoff, Dr. Herbert Mathews and Dr. Paula White for their helpful suggestions.

Additionally, I would like to thank all the members of the Iwashima Lab for their support and technical help. Special thanks to Dr. Markio Takami, Dr. Kathleen Jaeger, Stephanie Chapman and Jessica DeMaio. I would also like to thank my friends Jen Novak, Won Paik, Krystal White, Emily Field and Sheila McEwen for their support and friendship.

Finally, would like acknowledge Roy Triveline for sparking my interest in biology and Rong Yuan for the opportunity to gain experience in research. I would also like to thank my family Jim Krier, Karen Krier, Elizabeth Krier, Jeanette Krier, and William Burris for their continuous encouragement and motivation.

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LIST OF ABBREVIATIONS

ALK	Activin receptor-like kinase
APCs	Antigen presenting cells
CD	Cluster of differentiation
CpG	Cytosine phosphate guanine
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FC	Fragment, crystallizable
FKH	Forkhead domain
Foxp3	Forkhead box protein 3
GH	Growth hormone
Gp	Glycoprotein
GVHD	Graft-versus-host disease

H3K4me3	Histone 3 lysine 4 tri-methylation
H3K9ac	Histone 4 lysine 9 acetylation
H3S10ph	Histone 3 serine 10 phosphorylation
H4K8ac	Histone 4 lysine 8 acetylation
HEPES	4-(2-hydroxyethyl)-1 piperazineethanesulfonic acid
H-RS	Hodgkin's and Reed/Sternberg cells
IBD	Inflammatory bowel disease
IFN	Interferon
IgG	Immunoglobulin G
IL	Interleukin
IPEX	Immune dysregulation polyendocrinopathy, enteropathy, X-linked
iTreg cells	Inducible regulatory T cells
JAK2	Janus kinase 2
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis

Multi-CSF	Multi-lineage colony stimulating factor
nTreg cells	Natural regulatory T cells
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
RPMI 1640	Roswell park memorial institute 1640
Smads	Class of signal transducer proteins
STATs	Signal transducer and activator of transcription
TCR	T cell receptor
TGF- β	Transforming growth factor beta
Th	T helper
UCB	Umbilical cord blood
WCB	Whole cord blood

ABSTRACT

The immune system is a group of structures and processes that protect us from disease. In order to function properly it must recognize a wide variety of pathogens such as viruses and bacteria; but, must also distinguish these pathogens from the hosts own tissue. T cells play a crucial role in an immune response against these pathogens through many different mechanisms. However, an overactive immune response can lead to autoimmune diseases, therefore it is important that the immune system has the ability to negatively regulate an immune response and maintain homeostasis. In the periphery, regulatory T cells ($CD4^{+}CD25^{+}Foxp3^{+}$) are involved in the maintenance of self-tolerance and immune homeostasis.

Tregs express the transcription factor Foxp3 which is involved in the positive and negative regulation of multiple genes that are involved in Treg function. A mutation in the *foxp3* gene results in the fatal autoimmune disease Immunodysregulation Polyendocrinopathy Enteropathy X-linked syndrome (IPEX) in humans and Scurfy in mice. Treg dysfunction has also been associated with other diseases such as cancer, allergy, inflammatory bowel disease (IBD), multiple sclerosis (MS), and infection. Therefore, elucidating the mechanisms by which Tregs are induced may lead to advancements in the establishment of Treg based therapeutics for the aforementioned human diseases.

There are two main subsets of Tregs: naturally arising Tregs (nTregs) and inducible Tregs (iTregs). Natural Tregs develop in the thymus while iTregs differentiate from naïve $CD4^{+}$ T cells in the periphery. We have previously demonstrated that human umbilical cord blood (UCB) has an increased capacity for iTreg differentiation than human adult peripheral blood mononuclear cells (PBMCs). While effector $CD4^{+}$ T cells transiently up-regulate Foxp3 expression upon T cell receptor (TCR) stimulation, human Tregs stably express the transcription factor. Stable expression of Foxp3 is a marker for both nTregs and iTregs with functional suppressive capability.

Many mechanisms are involved in the induction of iTregs from naïve $CD4^{+}$ T cells which can include cytokine signaling as well as epigenetic modifications that control gene expression. These mechanisms may be utilized for the differentiation of iTregs by regulating the expression of genes required for the function of iTregs such as Foxp3; therefore, I investigated which epigenetic histone modifications and cytokines that may be involved in iTreg differentiation in an *ex vivo* human UCB model.

CHAPTER ONE

LITERATURE REVIEW

Introduction

The immune system is a collection of tissues and processes that protect us from pathogens such as bacteria, viruses and parasites; but must also distinguish pathogens from self-tissue. An adaptive immune response, or pathogen specific response, is orchestrated through the action of many immune cells including CD4⁺ T lymphocytes. CD4⁺ T cells can mediate an immune response through a variety of mechanisms which involves communication with other immune cells through cytokine and chemokine production. CD4⁺ T cells can also assist B cells in antibody production and macrophages by enhancing their activity to fight infections, thus the name T helper cell (Th). T cells express T cell receptors (TCR) on their cell surface capable of recognizing peptides derived from pathogens presented on major histocompatibility complex class II (MHC class II) that are expressed by antigen presenting cells (APCs). This interaction mediates downstream signaling through TCR that promote the transcription of many genes including cytokines and proteins that promote differentiation and function.

It has been established that T helper cell differentiation is very complex since many subsets have been reported inducing: Th1, Th2, Th9, Th17, and Tregs (1,2). Each of these subsets are characterized though specific function and cytokine production such

as interferon gamma (IFN γ) production for Th1 responses. All subsets must operate in concert in order for appropriate immune response to be mounted, the infection cleared, and discontinuation of the response along with establishment of memory. Autoimmunity occurs when an immune response is uncontrolled and has mounted a response against self-tissue; hence, the requirement to negatively regulate an inappropriate immune response is essential.

Regulatory T cells

Regulatory T cells mediate immune responses to maintain self-tolerance and immune homeostasis. Gershon and Kondo discovered a T cell subset that was able to diminish an immune response and were dissimilar from helper T cells (3). These cells, entitled suppressor T cells were continuously studied with results that fluctuated considerably. It was thought that the I-J molecule associated with MHC was supposed to elicit this suppressive function; however, this turned out to be untrue (4). Interest declined because no phenotypic markers could be determined until Sakaguchi discovered that CD4⁺CD25⁺ cells could down-regulate an immune response (5). CD25 is the interleukin 2 receptor α chain and is expressed on 5-10% of CD4⁺ T cells in the periphery (5). It has been established that CD25 expression and IL-2 signaling is essential for the proliferation and differentiation of Treg cells (6). Sakaguchi was able to transfer CD4⁺CD25⁻ or CD4⁺CD25⁺ T cells into lymphocyte deficient mice and mice receiving the CD4⁺CD25⁻ T cells developed apparent autoimmune disease while mice that received CD4⁺CD25⁺ cells did not (5). These data verify that Tregs have suppressive function

essential for self-tolerance and immune homeostasis and this function is dependent on interleukin 2 (IL-2).

It was not known what regulated this suppressive function until Bennett et al. found that mutations in the *foxp3* gene resulted in Scurfy mice, exhibiting autoimmune diseases including splenomegaly and lymphadenopathy, and the fatal autoimmune disease Immunodysregulation Polyendocrinopathy Enteropathy X-linked syndrome (IPEX) in humans (7). Further studies reported that Foxp3 (forkhead box P3) was specifically expressed in CD4⁺CD25⁺ cells and overexpression of Foxp3 in CD4⁺CD25⁻ non-Treg cells could generate cells with a Treg-like phenotype (9). These findings suggested that Foxp3 was the master regulator and CD4⁺CD25⁺Foxp3⁺ became the characteristic phenotype for Tregs. It has also been shown that CD8⁺Foxp3⁺ cells also have immunosuppressive capabilities (8). However, CD8⁺ Treg cells represent a novel Treg subset with findings still in its infancy. Foxp3 is a transcription factor that is a member of the forkhead/winged-helix family of transcriptional regulators and has a fork head (FKH) domain that binds to DNA thereby positively and negatively regulating gene expression important for Treg function (10).

Multiple mechanisms have been proposed for the suppressive function of CD4⁺CD25⁺Foxp3⁺ Treg cells. Hori et al. showed that Treg cells required antigenic stimulation through TCR signaling in order to suppress cells associated with an immune response (9). Cytokine production as well as APC interaction has also been proposed as a mechanism by which Tregs can down-regulate an immune response. Nakamura et al. has demonstrated that transforming growth factor beta (TGF- β) bound to the cell surface of

Tregs was also essential for Treg function (11). Oderup et al. showed that Treg interaction with dendritic cells (DCs) could down regulate the co-stimulatory molecule B7 on the DC cell surface; thus, inhibiting the activation of responder cells (12). These results suggest that Treg cells suppress an immune response through direct or indirect mechanisms.

There are two subsets of Tregs: natural Tregs (nTregs) that develop in the thymus and inducible Tregs (iTregs) that develop in the periphery (13). Naïve CD4⁺ T cells in the periphery can be stimulated to differentiate into iTregs through antigen presentation in the presence of TGF- β and these iTregs also express Foxp3; however, Foxp3 is not as stable in iTregs as it is in nTregs (13,14). It has been proposed that due to the variation in the way these two subsets develop, there may be dissimilar mechanisms of Foxp3 regulation (13). Many factors play a role in the generation of iTregs in the periphery which include exposure to antigen, cytokines, and APCs (13). All of these factors transmit signals throughout the cell which collectively determine and regulate the transcription of genes.

Epigenetics

Gene transcription can be regulated through an assortment of mechanisms including epigenetic modifications. Such modifications can include DNA methylation and histone modifications that can change the structure of DNA but not its sequence (15). This change in structure has downstream effects on the transcription of genes which may play a role in the differentiation of iTregs (14,15). DNA can be methylated at the

5' position of cytosine nucleotide when it occurs next to a guanine nucleotide (CpG) and DNA methyltransferases are involved in the maintenance of methylation during cell differentiation (15). Core histone molecules are organized into octamers consisting of two of each histone protein H2a, H2b, H3 and H4 and this octamer forms a complex with DNA known as a nucleosome (15). Modifications including acetylation, phosphorylation, methylation and ubiquitination, take place at highly conserved amino acid residues at the tail region of histone proteins and these modifications work together to influence the folded state of the chromatin (15). Open chromatin, or euchromatin, allows gene expression by permitting the binding of transcriptional machinery to the DNA at the promoter of a gene (15).

Floess et al. has demonstrated that both DNA methylation and histone modifications are mechanisms by which Foxp3 is regulated in Tregs (14). CpG motifs within amplicons 1 and 2 showed a high degree of methylation on CD4⁺CD25⁻ conventional T cells; whereas, in CD4⁺CD25⁺ Tregs this region was almost completely de-methylated which permits transcription (14). No changes in the methylation status between amplicon 3 and 4 suggests that this change in methylation is not random (14). Floess et al. also demonstrates that pro-transcriptional modifications tri-methylation of histone protein H3 at lysine residue 4 (H3K4me3) and acetylation of histone protein H3 (H3ac) at the Foxp3 locus has a stronger association with CD4⁺CD25⁺ Tregs when compared to CD4⁺CD25⁻ conventional T cells (14). These data suggests that DNA methylation and histone modifications are vital for the expression of Foxp3 and function of Tregs.

Cytokines signaling

Cytokine milieus are an important factor in cell signaling and are a key regulator in the differentiation of all immune cells. These small proteins bind to their cognate receptors and mediate an intracellular signaling cascade that results in alteration of gene expression. Classification of receptors is based upon the extracellular structure. This includes the IL-2 receptor family, growth hormone (GH) family, interferon (IFN) family, glycoprotein 140 (gp140) family and TGF- β receptor family, among many others (16). Cytokine receptors are vital as they are responsible for receiving a signal through cytokine binding and are also responsible for transmitting that signal to downstream target proteins.

TGF- β is a pleiotropic cytokine that binds to the TGF- β receptor and facilitates proliferation, differentiation and apoptosis of many cells and can be produced by many cells types including lymphocytes and macrophages (17). TGF- β has 3 isoforms; TGF- β 1, 2, and 3, and has been implicated in many diseases such as cancer, heart disease, diabetes, and AIDS (18). It has been established that TGF- β is a critical factor in T cell immune homeostasis as a T cell specific deletion of the TGF- β receptor II in mice results in lethal inflammation (20,19). Intracellular transducers that are activated upon TGF- β stimulation include Smads and Ross et al has shown that Smad2 mediated gene transcription requires the histone acetyltransferase p300 for acetylation of H3K9 at promoter regions to promote gene expression (21). These data support the idea that TGF- β signaling is required for regulating T cell immune homeostasis and regulation of

gene transcription; furthermore, Smads depend on histone remodeling to promote gene expression.

It is known that regulatory T cells are required for peripheral tolerance and Li et al. has shown that the number of Tregs declines when TGF- β receptor II is deleted in T cells; however, the number of Tregs in the thymus was unchanged (19). This demonstrates the importance of TGF- β to the peripheral maintenance of iTregs. Furthermore, Chen et al demonstrated that naïve CD4⁺ T cells were able to differentiate into Foxp3⁺ iTregs in the periphery with TCR stimulation in the presents of TGF- β (22). The expression of Foxp3 is mediated by TGF- β activation of Smad3 which translocates to the nucleus and associates with the *foxp3* promoter (23). These data indicate that TGF- β is required for the differentiation of iTregs in the periphery through the regulation of chromatin structure and gene expression important for iTreg function.

Interleukin-3 (IL-3), also known as multi-lineage colony stimulating factor (Multi-CSF), is a cytokine that is secreted by activated T cells and can stimulate the proliferation and differentiation of pluripotent hematopoietic stem cells (24,25). IL-3 is also capable of promoting growth and differentiation of neutrophils, macrophages, lymphoid and erythroid cells by binding to the IL-3 receptor which is a member of the gp140 family (26). Treg dysfunction has been implicated in many autoimmune diseases and mounting evidence has linked autoimmunity to cancer; additionally, dysfunction in IL-3 signaling may play a role in immune imbalances that are associated with these diseases (27). One example is classical Hodgkin's disease which is a form of lymphoma characterized by low tumor frequency Hodgkin's and Reed/Sternberg cells (H-RS) (28).

Aldinucci et al. has shown that these H-RS cells express the IL-3 receptor and exogenous IL-3 can promote the growth and inhibit apoptosis of these cells (28). IL-3 is important for the differentiation of hematopoietic stem cells, thus it is important for the generation of all cell types found in the blood; however, under inappropriate circumstances can allow proliferation and anti-apoptotic signals to tumor cells.

An increasing amount of evidence suggests that IL-3 may play a role in the differentiation of naïve CD4⁺ T cells to iTregs in the periphery. Seivastava et al. has demonstrated that the IL-3 receptor is expressed by iTregs and IL-3 enhances the frequency of Foxp3⁺ T cells through the indirect secretion of IL-2 by non-Treg cells (25). Signal transducers and activators of transcriptions (STATs) are activated when IL-3 binds to its cognate receptor (25,26,29). Nakajima et al shows that STAT5^{-/-} splenocytes have a decrease in IL-2 receptor α chain/CD25 mRNA compared to wild type cells (30). Furthermore, Antov et al determined that Treg number and function is defective in STAT5 knockout mice (29). These data propose that IL-3 may be important for iTreg differentiation and proliferation through the activity of STAT5.

Proposed model

We have previously discovered that an *ex vivo* human umbilical cord blood (UCB) culture system has an increased capacity for the differentiation of iTregs than adult peripheral blood mononuclear cells (PBMCs). However, it is not completely understood what mechanisms and signals are required for the differentiation of iTregs from UCB. Cytokine signaling and epigenetic modifications of histone tail residues are

well known mechanisms that regulate gene expression. Hence, we propose a model that iTreg differentiation from human UCB utilizes histone modifications mediated through TGF- β signaling as well as IL-3 signaling, a novel cytokine for iTreg proliferation, differentiation and cell survival.

CHAPTER TWO

MATERIALS AND METHODS

Isolation of mononuclear cells from umbilical cord blood

Human umbilical cord blood (UCB) was collected at Gottlieb Memorial Hospital without any identifying information. UCB samples were collected into sterile, anticoagulant treated BLOOD-PACK[™] units (Fenwal, Inc., IL) and were diluted 1:1 with 1x PBS at room temperature. Mononuclear cells were then enriched by ficoll-hypaque density gradient centrifugation using lymphocyte separate media (Cellgro). The buffy coat layer containing mononuclear cells was then extracted by pipetting. The procedure was repeated to reduce red blood cell contamination. Mononuclear cells are collected, counted, and subjected to flow cytometry or cell culture. Total CD4⁺ T cells were enriched from whole UCB (WCB) mononuclear cells via BD IMag Enrichment Sets (Becton Dickinson) according to the protocol provided by the manufacturer.

Culture Media

RPMI 1640 media (Thermo Scientific), supplemented with 10% FCS (Atlanta Biologicals), β -mercaptoethanol (50 μ M), glutamine (2 mM), penicillin (100 I.U./mL), streptomycin (100 μ g/mL), HEPES (10 mM), sodium pyruvate (1 mM), and MEM essential and non-essential amino acids (Invitrogen) were used in the experiments.

Cell Culture

Cells were cultured in 48 well plates in culture media. Cultures were supplemented with human IL-2 (PeproTech) at 10 ng/mL and α -CD3 (eBiosciences) at 0.2 μ g/mL. Cells were split every 2-3 days and IL-2 concentrations were maintained throughout culture. An inhibitor of TGF- β signaling, SB-431542 (Sigma-Aldrich) was added at 10 μ M, anti-TGF- β (1,2,3) mouse mAb (R&D systems) was added at 2.5 ng/mL and recombinant human TGF- β (R&D systems) was added at a concentration of 2.5 ng/mL where indicated. Human recombinant IL-3 (PeproTech) was added to culture at a concentration of 10 ng/mL and purified human anti-IL-3 (Biolegend) was added to culture at 1 μ g/mL. TGF- β inhibitor SB-431542, anti-TGF- β (1,2,3), recombinant TGF- β , recombinant IL-3 and anti-IL-3 concentrations were all maintained throughout culture.

Polystyrene beads were incubated with 10 μ g α -CD3 and 10 μ g α -CD28 per 100 μ l bead suspension overnight at room temperature. Beads were then washed with 1x PBS and blocked in 1x PBS with 10% BSA for 2 hours. After blocking, beads were re-suspended in cell culture media to 2×10^6 beads per 50 μ l media. For stimulation, 1×10^6 CD4⁺ cells were incubated with 50 μ l beads for 1 hour at 37°C before transferring to cell culture plate.

Flow Cytometry

Fluorochrome-conjugated antibodies specific for CD3 (clone UCHT1), CD4 (clone RPA-T4), CD14 (clone HCD14), CD25 (clone BC96), CD36 (clone 5-271), and CD123 (clone 6H6) (Biolegend) CD8 (clone RPA-T8) and Foxp3 (clone 259D/C6)

(BD Biosciences) were used in the experiments. Cells were washed in FACS buffer (1% FCS, 0.1% sodium azide in 1x PBS) and then incubated with purified human IgG (R&D systems) for FC receptor blocking. Cells were then washed with FACS buffer and stained with the indicated antibodies at 4°C for 30 minutes. For Foxp3 staining, cells were fixed and permeabilized using FOXP3 staining buffer set (Biolegend). Cells were washed twice with 1x PBS after cell surface staining followed by fixation with 500 µl fix/perm buffer for 20 minutes at room temperature. Tubes were then filled with FACS buffer and kept overnight at 4°C. The next day cells were washed with FACS buffer and incubated in 1x permeabilization buffer for 15 minutes at room temperature. Cells were then stained with anti-Foxp3 antibody for 30 minutes at room temperature followed by washing with FACS buffer. Data was collected on a flow cytometer (FACS Canto II, BD Biosciences) and analyzed using FlowJo software (TreeStar, Inc.).

Histone Staining

Cells are harvested and washed with FACS Buffer and incubated with purified human IgG for FC receptor blocking. After surface staining, cells are washed twice with FACS buffer followed by fixation and permeabilization with cytofix/cytoperm solution (BD Biosciences). Cells are then washed twice with perm/wash buffer (BD Biosciences). Phosphor-histone H3 (Ser10) (Alexa Fluor 488 conjugate), acetyl-histone H3 (Lys9) (C5B11) rabbit mAb (Alexa Fluor 488 conjugate), tri-methyl-histone H3 (Lys4) (C42D8) rabbit mAb (Cell Signaling), and anti-acetyl histone H4 (Lys8) (Millipore) are added and incubated at 4°C for 1 hour. Cells are washed twice with perm/wash buffer. Goat

anti-rabbit IgG (H+L) fluorescein conjugated antibody (Millipore) was added as a secondary for H3K4 and H4K8 antibodies and incubated at 4°C for 30 minutes. Cells are washed with perm/wash buffer and re-suspended in 200 µl FACS buffer. Data was collected on a flow cytometer (FACS Canto II, BD Biosciences) and analyzed using FlowJo software (TreeStar, Inc).

CHAPTER THREE

EXPERIMENTAL RESULTS

Global Histone Modifications in CD4⁺ Treg cells and CD4⁺ non-Treg cells

Since iTregs (CD4⁺CD25⁺Foxp3⁺) express different genes than non-Treg T cells (CD4⁺CD25⁻Foxp3⁻), it is expected that Tregs use epigenetic modifications to control gene expression. During transcription, histone modifications are used to control the folded state of chromatin, therefore blocking or promoting gene transcription, and this alteration is controlled through an assortment of histone-modifying enzymes. Although, a variety of modifications are needed for full activation and repression of certain genes, a few important modifications that promote gene expression include H3K4 tri-methylation (me3), H3K9 acetylation (ac), H3S10 phosphorylation (ph) and H4K8 acetylation (ac). Floess et al. has shown that nTregs utilize H3 acetylation and H3K4me3 to regulate expression of Foxp3 (14). Therefore, I hypothesized that the aforementioned histone modifications are globally altered in iTregs (CD4⁺CD25⁺Foxp3⁺) compared to non-Treg cells (CD4⁺CD25⁻Foxp3⁻) from UCB. Enriched CD4⁺ T cells stimulated with α -CD3 and α -CD28 coated beads with the addition of exogenous IL-2 do not induce iTreg differentiation and therefore will be the non-Treg (CD4⁺CD25⁻Foxp3⁻) control. First, we determined the Treg frequency of whole UCB (WCB) culture as well as CD4⁺ T cells simulated with beads by fluorescence activated cell sorting (FACS) after fourteen

days of culture (Figure 1). Tregs are characterized by the expression of the IL-2 receptor α chain (CD25) and the transcription factor Foxp3. CD4⁺ cells from WCB expressed the highest levels of CD25 and Foxp3 while CD4⁺ cells after bead stimulation expressed very little Foxp3 with moderate CD25 expression as activated T cells do express CD25.

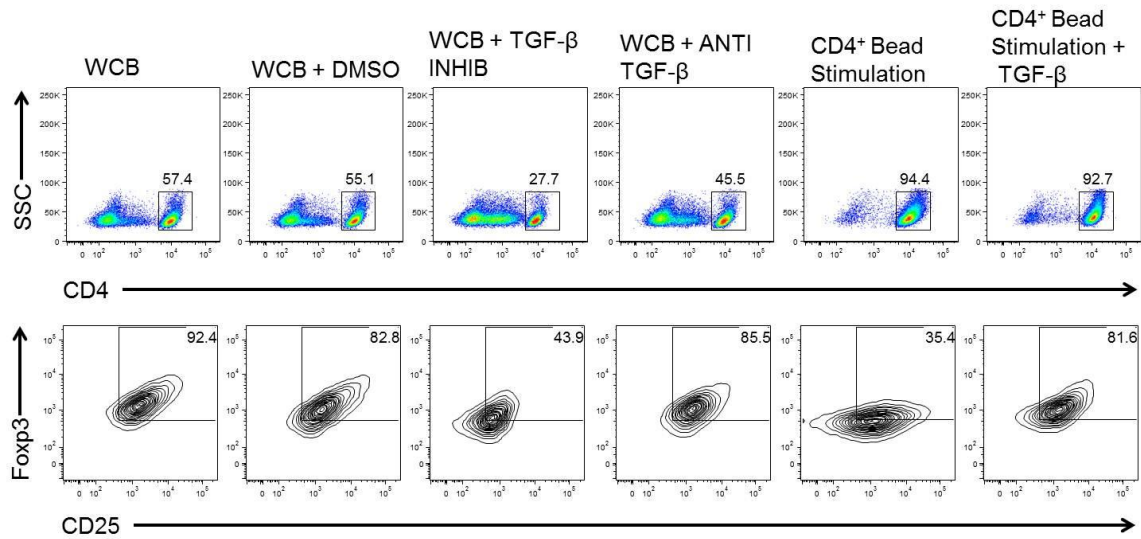


Figure 1: FACS analysis of Treg frequency in *ex vivo* UCB. WCB cultures are stimulated with α -CD3 and IL-2 with or without the addition TGF- β inhibitor SB-431542 or anti-TGF- β neutralizing antibody. DMSO treated WCB is used as a control. CD4⁺ cultures are stimulated with α -CD3 and α -CD28 coated polystyrene beads with the addition of IL-2 with or without exogenous TGF- β . Cells were harvested at day 14 and stained for CD4, CD25 and Foxp3. Cells are gated on CD4⁺ (Top panel) and Treg frequency is determined by CD25 and Foxp3 expression (Bottom panel).

To determine if histone modifications are altered in iTreg cells compared to non-Treg cells, WCB cells were cultured for fourteen days to induce iTreg differentiation with α -CD3 and IL-2. For non-Treg cultures, CD4⁺ cells were stimulated with beads as previously described. Cells were harvested and the histone modifications H3K4me3, H3K9ac, H3S10ph and H4K8ac was determined by FACS (Figure 2). These modifications are involved in remodeling the chromatin structure into euchromatin, or lightly packed chromatin, near promoter regions to allow binding of transcriptional machinery, thus promoting transcription. A decrease was observed for H3K4me3 and H3K9ac in CD4⁺ iTregs from WCB compared to CD4⁺ non-Tregs from bead stimulated cultures; however, no difference was seen in the level of H3S10ph and H4K8ac. The mean fluorescence intensity (MFI) also recapitulates this finding (Figure 2). At day five of culture, the decrease in H3K4me3 and H3K9 is apparent (Figure 3). This suggests that H3K4me3 and H3K9ac are being globally reduced to stop any unnecessary gene transcription while only genes involved in iTreg differentiation and function may be transcribed. H3S10ph and H4K8ac modifications are present within iTregs; however, their role may be important for transcription of housekeeping genes that are important for the survival of all cell types.

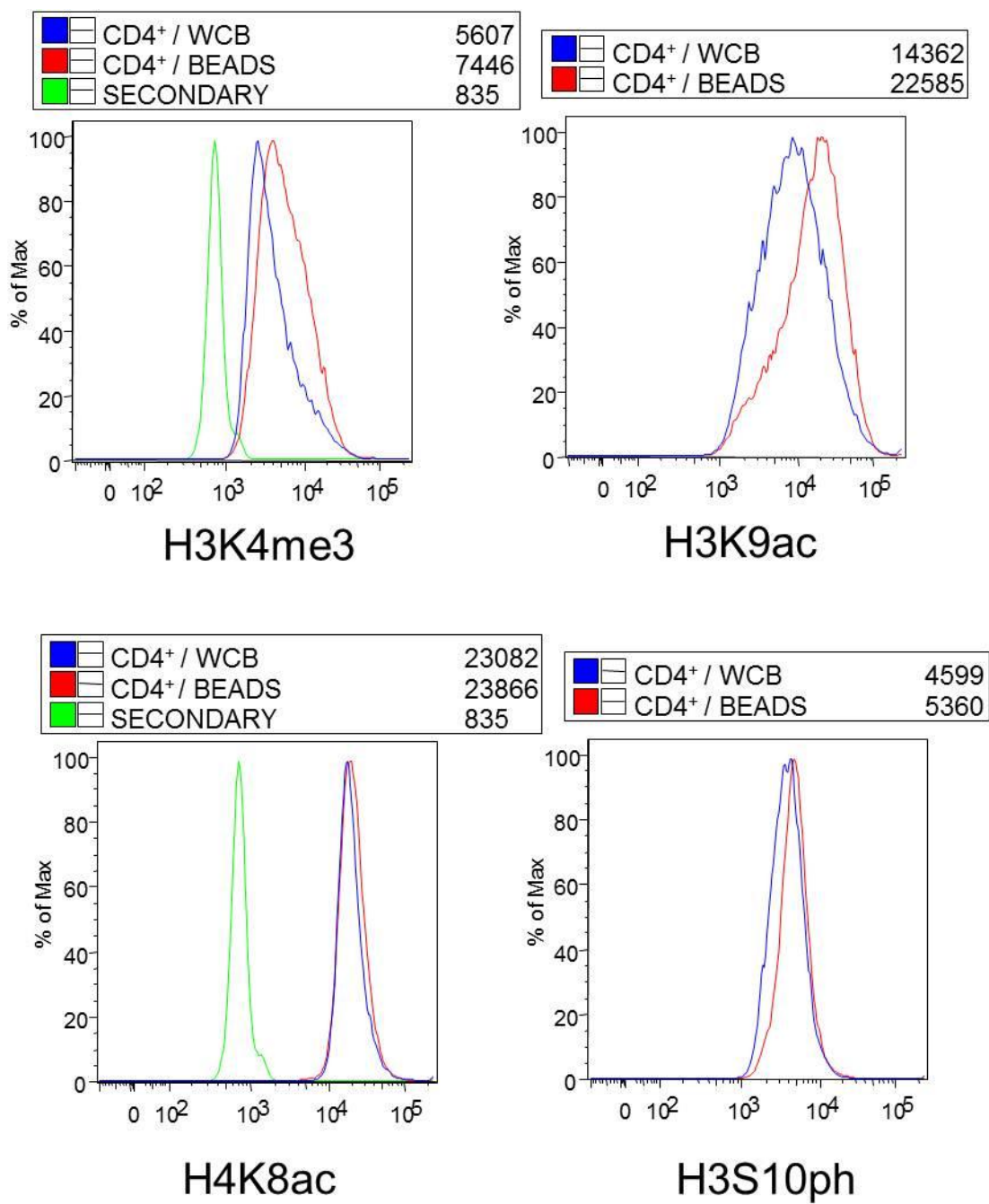
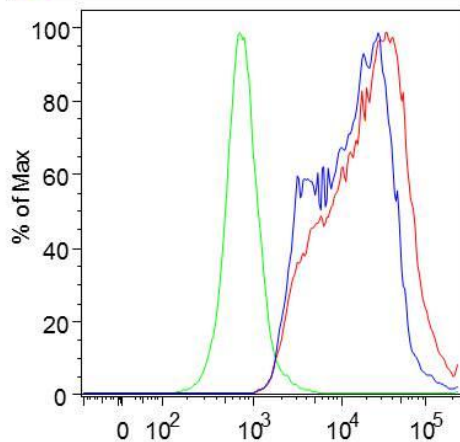
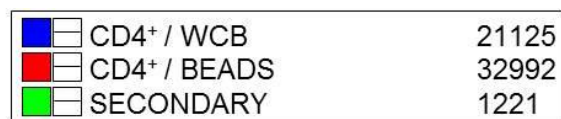
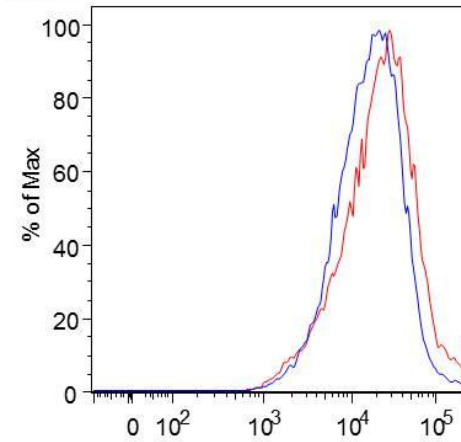
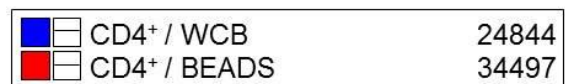


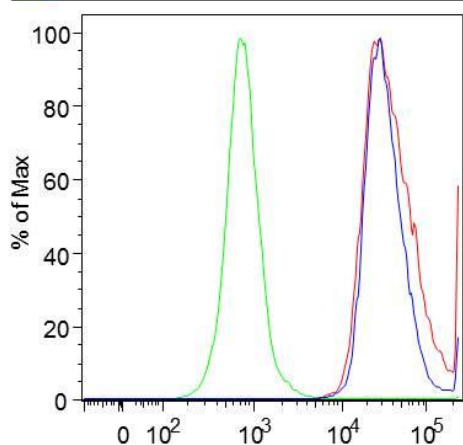
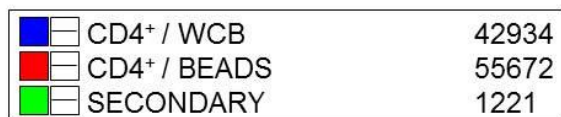
Figure 2: FACS analysis of histone modifications in Treg cells vs. Non-Treg cells at Day 14. WCB is stimulated with α -CD3 and IL-2. CD4⁺ cells are stimulated with α -CD3 and α -CD28 coated polystyrene beads with the addition of IL-2. Cells are harvested at day 14 and stained with CD4, H3K4me4, H3K9ac, H4K8ac and H3S10ph. Cells are gated on CD4⁺ and then histone modifications are analyzed by histogram. MFI is reported above.



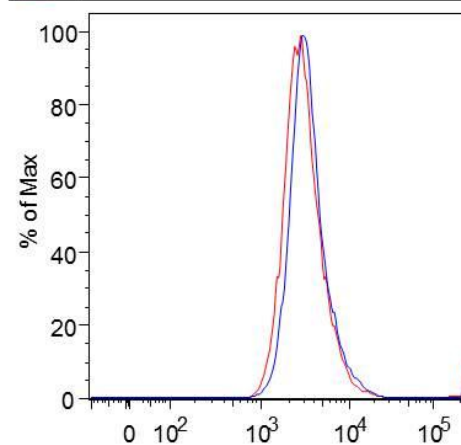
H3K4me3



H3K9ac



H4K8ac



H3S10ph

Figure 3: FACS analysis of histone modifications in Treg cells vs. Non-Treg cells at Day 5. WCB is stimulated with α -CD3 and IL-2. CD4⁺ cells are stimulated with α -CD3 and α -CD28 coated polystyrene beads with the addition of IL-2. Cells are harvested at day 5 and stained with CD4, H3K4me4, H3K9ac, H4K8ac and H3S10ph. Cells are gated on CD4⁺ and then histone modifications are analyzed by histogram. MFI is reported above.

TGF- β signaling is important for global alterations in histone modifications

We have demonstrated that H3K4me3 and H3K9ac have decreased in Treg cells compared to non-Treg cells. One cytokine that has been shown to induce iTregs from naïve CD4⁺ T cells is TGF- β (22). Many cells, such as monocytes, can secrete TGF- β and it has been postulated that membrane bound TGF- β is present and important in our culture system. Activated TGF- β binds to the TGF- β receptor II and forms a heterodimer with the TGF- β receptor I which is required for kinase activity (31). Upon dimerization, the serine/threonine kinase domain phosphorylates a serine residue on TGF- β receptor I which mediates downstream signaling through the transducer proteins Smad2 and Smad3. Activated Smad2/3 have a high affinity for binding a co-Smad, Smad4, and this heterodimer complex translocates to the nucleus and can bind DNA and regulate gene expression (31). Ross et al. has shown that Smad2 requires the histone acetyltransferase, p300, to acetylate H3K9 in order to alter chromatin assembly at target genes (21).

Next, we assessed if TGF- β signaling was important for modulating the decrease in global H3K4me3 and H3K9ac seen in iTreg cells from WCB. Since TGF- β is absent in

our CD4⁺ bead simulated non-Treg culture, exogenous TGF- β was added to culture. We hypothesized that iTreg frequency would be rescued and that H3K4me3 and H3K9ac would be similar to CD4⁺ iTreg cells from WCB. An increase in Treg frequency was seen in CD4⁺ bead stimulated cells when exogenous TGF- β was added when compared to CD4⁺ bead stimulated cells without TGF- β (Figure 1). Furthermore, H3K4me3 and H3K9ac are decreased in CD4⁺ bead stimulated cells with the addition of TGF- β similar to CD4⁺ iTreg cells from WCB. This observation was also reiterated by the decrease in the MFI seen in CD4⁺ bead simulated cells with the addition of exogenous TGF- β compared to CD4⁺ bead stimulated cells without TGF- β (Figure 4).

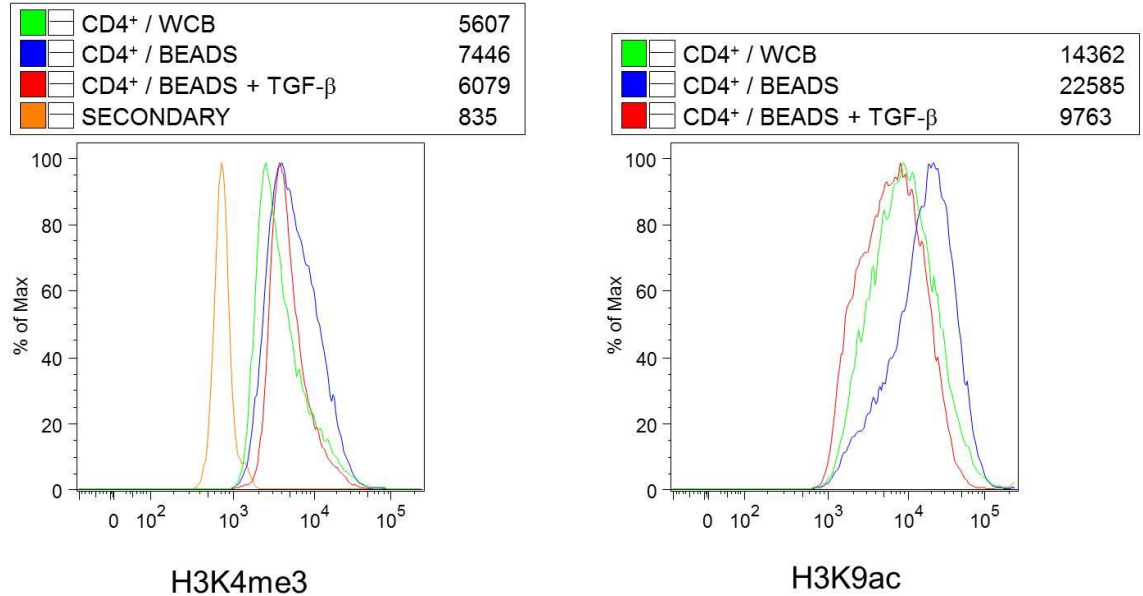


Figure 4: Effects of exogenous TGF- β addition to CD4⁺ bead stimulated culture on histone modifications. WCB is stimulated with α -CD3 and IL-2. CD4⁺ cells are stimulated with α -CD3 and α -CD28 coated polystyrene beads plus IL-2 with or without the addition of exogenous TGF- β . Cells are harvested at day 14 and stained with CD4, H3K4me4 and H3K9ac. Cells are gated on CD4⁺ and then histone modifications are analyzed by histogram. MFI is also reported.

We subsequently sought to determine if the inhibition of TGF- β signaling in our WCB culture system would affect H3K4me3 and H3K9ac. We hypothesized that if TGF- β is required for the modulation of H3K4me3 and H3K9ac, than inhibiting TGF- β in our WCB culture system would increase the H3K4me3 and H3K9ac compared to untreated WCB and reduce Treg frequency. Both the TGF- β SB-431542 small molecule

inhibitor and an anti-TGF- β (1,2,3) neutralizing antibody were used in the experiments. The TGF- β inhibitor SB-431542 blocks the activity of the TGF- β superfamily type I activin receptor-like kinase (ALK4, ALK5 and ALK7) which forms a complex with TGF- β receptor II and mediates the downstream signaling of TGF- β (32). The anti-TGF- β (1,2,3) antibody neutralizes any TGF- β (1,2,3) present in culture by blocking the ability to induce its biological effects. CD4⁺ cells from WCB cultures treated with TGF- β inhibitor or neutralizing antibody had a decrease in CD25 and Foxp3 expression compared untreated WCB; although, anti-TGF- β antibody did not have as great of an effect (Figure 1). Alternatively, no difference was detected in H3K4me3 and H3K9ac in CD4⁺ cells from WCB treated with TGF- β inhibitor SB-431542 or anti-TGF- β antibody compared to untreated WCB (Figure 5). When WCB was treated with TGF- β inhibitor SB-431542 and anti-TGF- β antibody, an increase in the frequency of CD4^{low} cells was observed (Figure 5A). We then determined if H3K4me3 and H3K9ac was increased in the CD4^{low} and CD4⁻ populations. A slight increase in H3K4me3 and H3K9ac was seen in the CD4^{low} and the CD4⁻ populations when compared to untreated WCB. This increase is represented in the MFI when comparing untreated WCB to WCB treated with either the SB-431542 inhibitor or the anti-TGF- β antibody (Figure 5B). These data suggest that TGF- β may be playing a role in the orchestration of chromatin remodeling in order to regulate gene expression for the differentiation of iTregs from UCB.

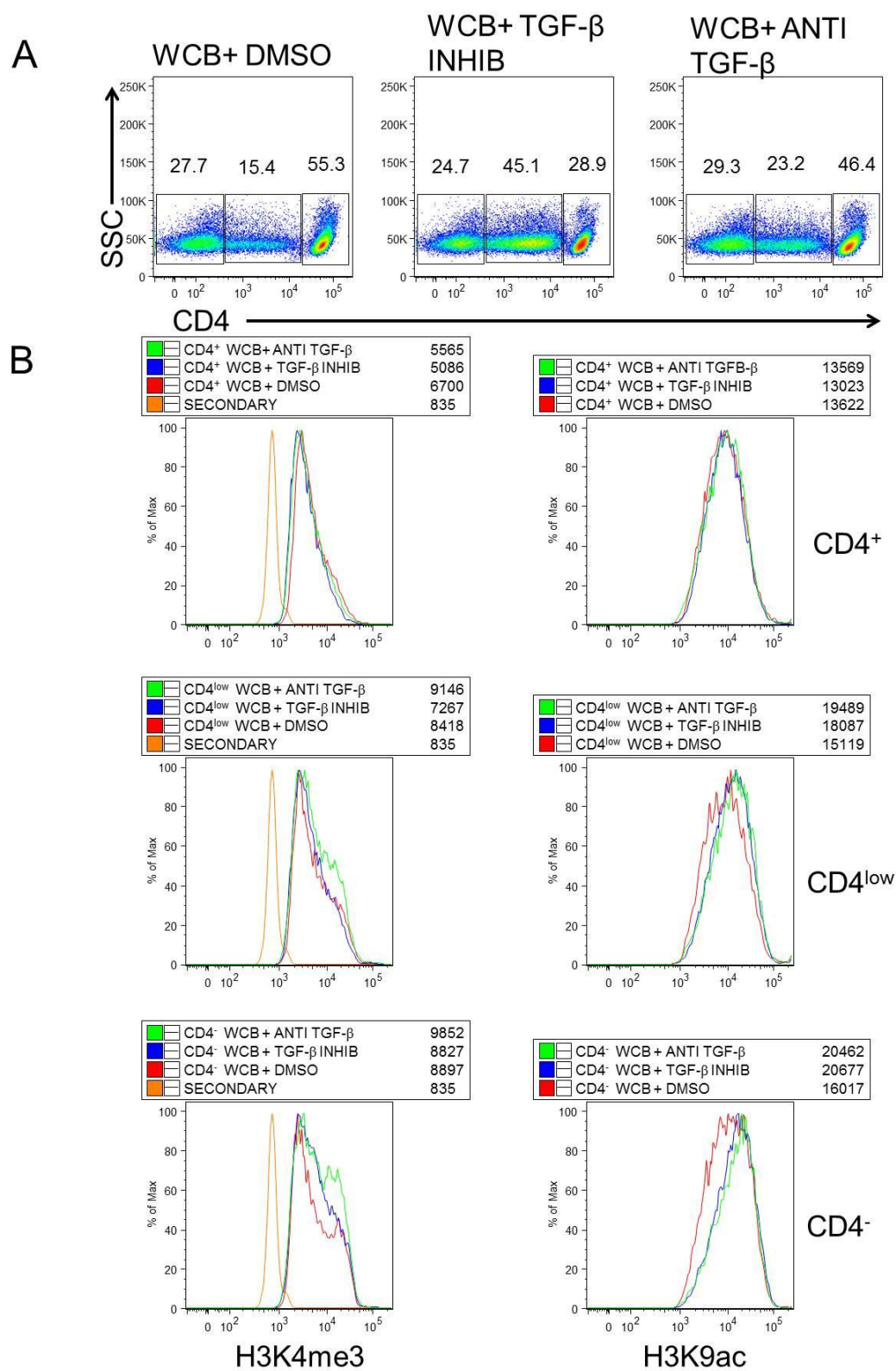


Figure 5: Effects of TGF- β inhibition of WCB on histone modifications. WCB is stimulated with α -CD3 and IL-2 with or without the addition of either TGF- β inhibitor SB-431542 or anti-TGF- β antibody. Cells are harvested at day 14 and stained with CD4, H3K4me4 and H3K9ac. Cells are gated on CD4⁺ CD4^{low} or CD4⁻ (A) and then histone modifications are analyzed by histogram (B). MFI is also reported.

CD4^{low} cells, surface markers, and Treg frequency of whole UCB

We next wanted to determine what these CD4^{low} cells were and if they were iTreg cells expressing CD25 and Foxp3. Monocytes express CD4 at low levels and can differentiate into plasmacytoid dendritic cells (pDC) (33). Stimulation of pDCs has been shown to induce CD4⁺CD25⁺ iTregs that are capable of inhibiting proliferation of autologous or allogeneic naïve CD4⁺ T cells in an antigen non-specific manner (34). An identifying surface marker for pDCs is CD123, the IL-3 alpha chain receptor (34). Therefore, we hypothesized that these CD4^{low} cells are either iTreg cells or monocytes that were differentiating into plasmacytoid dendritic cells (pDCs).

To address this question, we stained un-stimulated cells at day zero for both T cell and monocyte surface markers to analyze the cells that are present in the starting material. TGF- β inhibitor SB-431542 was used in these experiments due to the increased frequency in CD4^{low} cells observed previously. After fourteen days of culture with α -CD3 and IL-2 stimulation, with or without TGF- β inhibitor SB-431542, the cells were stained again for the same surface markers. Treg frequency was also determined by FACS at day fourteen. At day zero, CD3⁺ T cells express either CD4 or CD8 and monocytes in UCB

express the surface markers CD14 and CD36 (Figure 6A). It has been previously shown in the lab that these monocytes have an increased propensity for the generation of iTregs. These monocytes also express CD4 at low levels (Figure 4B). Interestingly, $CD14^+CD36^{hi}$ monocytes are $CD123^+$ while our $CD3^+$ T cells are $CD123^-$ (Figure 6B).

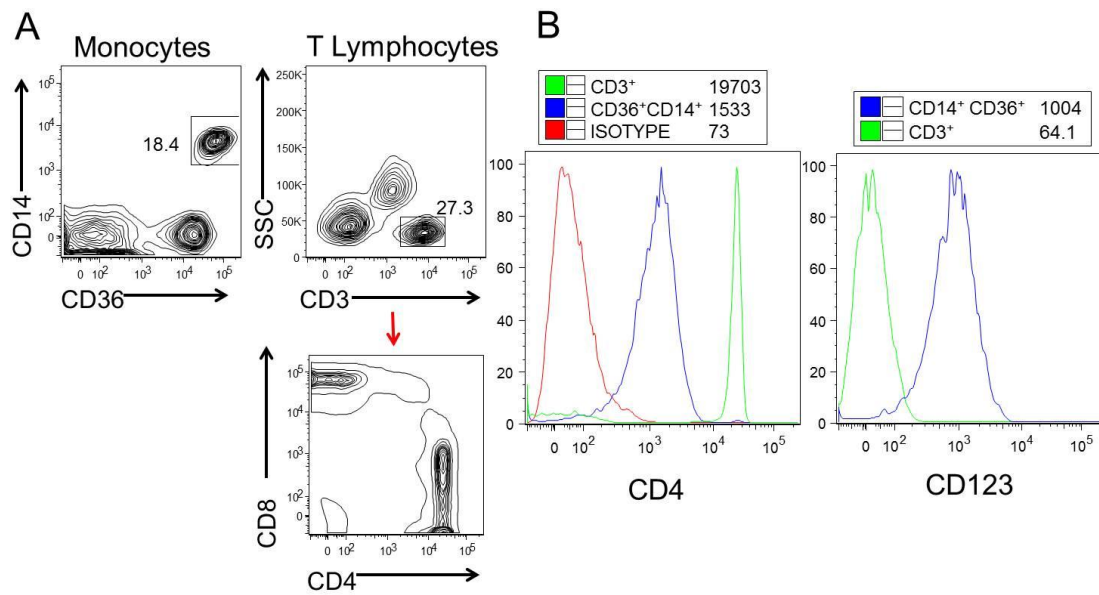


Figure 6: Cell surface expression at Day 0. WCB is un-stimulated and stained with CD3, CD4, CD8, CD14, CD36 and CD123. Monocytes are gated on $CD14^+CD36^{hi}$ cells and T lymphocytes are gated on $CD3^+$ cells. Expression of CD4 and CD8 is then views from $CD3^+$ cells (A). Expression of CD4 and CD123 is viewed for $CD3^+$ T lymphocytes and $CD14^+ CD36^{hi}$ monocytes (B).

After fourteen days of culture, cells were harvested and both surface staining and Treg staining was completed. All cells after fourteen days of culture are CD3⁺ T cells; thus, the CD4^{low} cells are not pDCs. The increase in the frequency of CD4^{low} cells was observed with TGF- β inhibition; however, these cells are also CD8⁺. Sullivan et al has shown that up-regulation of CD4 on CD8⁺ cells results in the increased expression of activation markers such as CD95, CD28, and CD25 when compared to CD8⁺CD4⁻ cells suggesting that CD4 expression on CD8⁺ cells is an additional marker to identify activated CD8⁺ T cells (35). It has also been shown that populations of CD8⁺Foxp3⁺ Tregs are vital for protection against graft-versus-host disease (GVHD) (8). However, CD8⁺ Tregs have not been well characterized in our UCB model. We analyzed the Treg frequency of each of the populations in the CD4 and CD8 profile. Cells that are CD4⁺ and express CD8 at low levels had an increase in Treg frequency when compared to CD4⁺CD8⁻ cells. The same was also seen in cells that are CD8⁺ and express CD4 at low levels when compared to cells that are CD8⁺CD4⁻ (Figure 7). Foxp3 and CD25 expression is also increased in CD8⁺CD4^{low} and CD4⁺CD8^{low} compared to CD8⁺CD4⁻ and CD4⁺CD8⁻ respectively (Figure 8). This suggests that these CD8⁺CD4^{low} and CD4⁺CD8^{low} cells are a more activated population of iTregs. Cultures with TGF- β small molecule inhibitor treatment show a flattened shape when looking at CD25 and Foxp3 expression in all populations signifying a decrease iTreg differentiation (Figure 7). Surprisingly, we also observed that the CD4⁺CD8⁻ and CD4⁺CD8^{low} cells expressed CD123 at day fourteen while CD8⁺CD4⁻ and CD8⁺CD4^{low} cells do not (Figure 9).

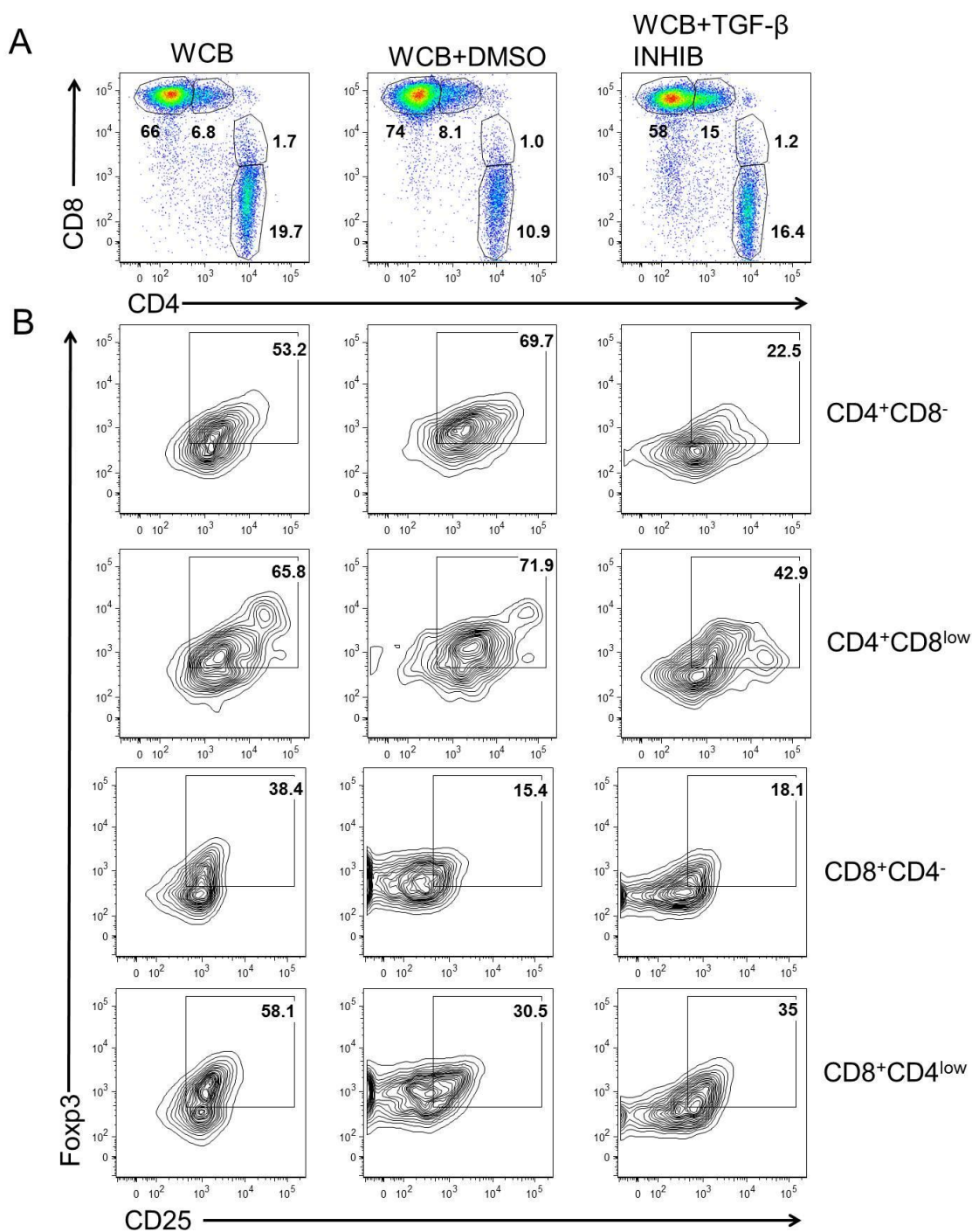


Figure 7: Treg frequency of CD4/CD8 populations in WCB. WCB cultures are stimulated with α -CD3 and IL-2 with or without the addition of TGF- β inhibitor SB-435142. DMSO treatment is used as a control. Cells were harvested at day 14 and stained for CD4, CD8, CD25 and Foxp3. Cells are gated on CD4⁺CD8⁻, CD4⁺CD8^{low}, CD8⁺CD4⁻ and CD8⁺CD4^{low} (A). Treg frequency is determined by CD25 and Foxp3 expression (B).

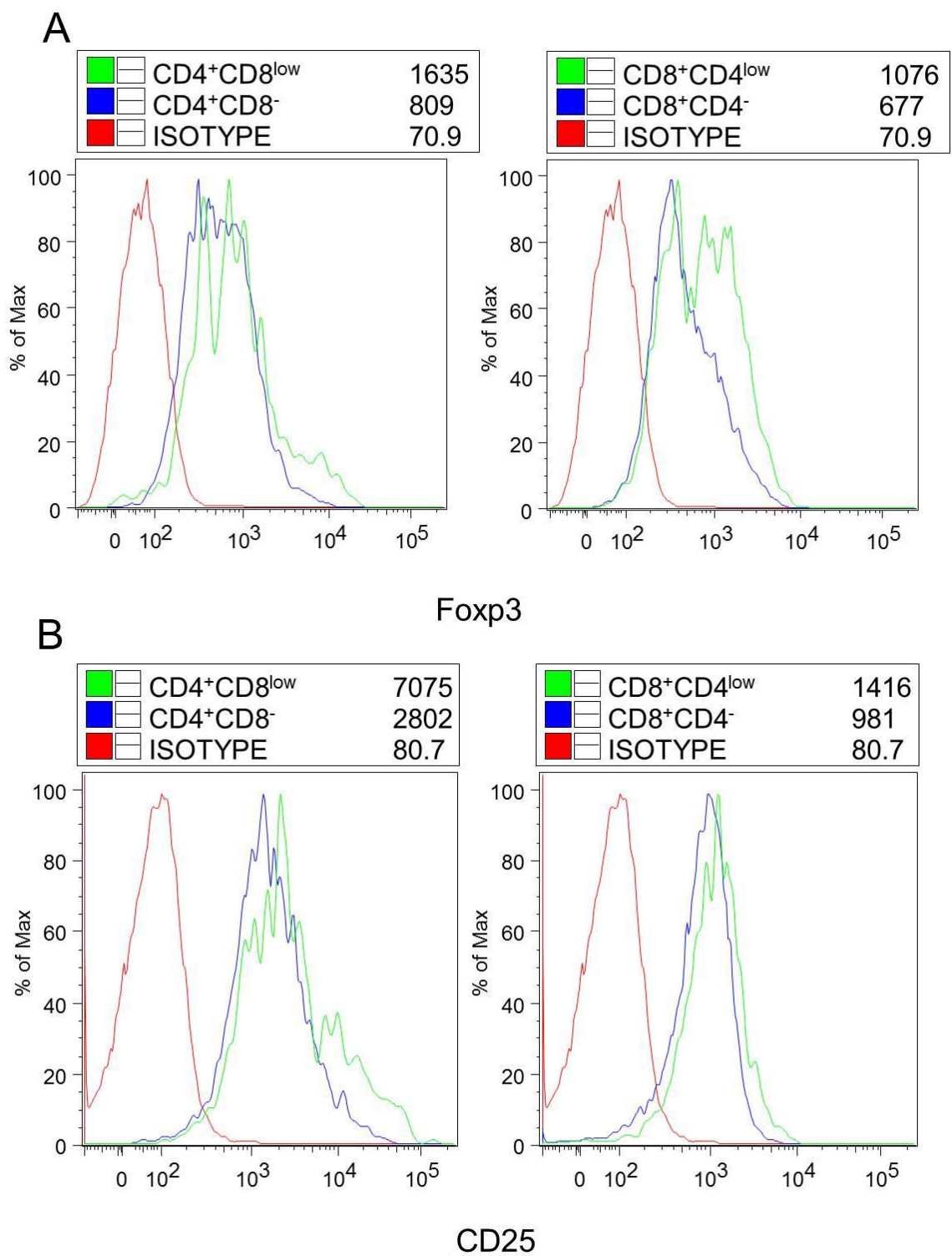


Figure 8: Foxp3 and CD25 expression in $CD4^+CD8^-$ vs. $CD4^+CD8^{low}$ and $CD8^+CD4^-$ vs. $CD8^+CD4^{low}$. WCB cultures are stimulated with α -CD3 and IL-2. Cells were harvested at day 14 and stained for CD4, CD8, CD25 and Foxp3. Foxp3 expression is observed in $CD4^+CD8^-$ vs. $CD4^+CD8^{low}$ and $CD8^+CD4^-$ vs. $CD8^+CD4^{low}$ (A). CD25 expression is observed in $CD4^+CD8^-$ vs. $CD4^+CD8^{low}$ and $CD8^+CD4^-$ vs. $CD8^+CD4^{low}$ (B).

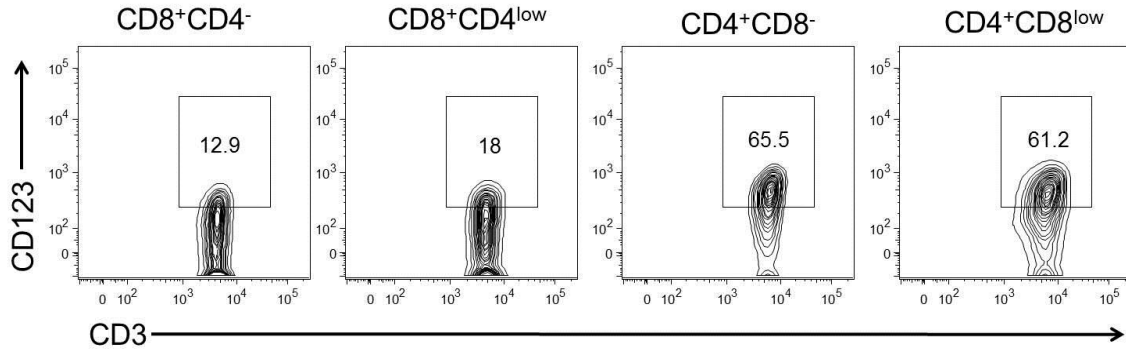


Figure 9: CD123 expression on CD4/CD8 populations. WCB cultures are stimulated with α -CD3 and IL-2. Cells were harvested at day 14 and stained for CD3, CD4, CD8, and CD123. Cells are gated on CD4⁺CD8⁻, CD4⁺CD8^{low}, CD8⁺CD4⁻ and CD8⁺CD4^{low} and CD3 and CD123 expression is observed.

IL-3 signaling in iTreg induction

We next wanted to determine if CD123 expression was functional and if IL-3 signaling was playing a role in iTreg induction. The populations described above expressed CD123 differently and this dissimilarity in expression may be relevant to the differentiation of CD4⁺ iTregs from UCB. Therefore, we hypothesized that IL-3 signaling may be influential in CD4⁺ iTreg differentiation. After IL-3 binds to its high affinity receptor IL-3R α (CD123), CD123 then forms a complex with a common beta chain, IL-3R β c (26). A tyrosine protein kinase, Janus kinase 2 (JAK2), binds to the cytoplasmic region of CD123, thus activating JAK2 which sequentially activates IL-3R β c (36). IL-3R β c now serves as a docking station to activate the signal transducer proteins STATs,

such as STAT5 which forms a dimer, enters the nucleus, and regulates gene transcription (26). Srivastava et al. has demonstrated that IL-3 signaling through STAT5 increases the frequency of functional Foxp3⁺ Treg cells (25).

In order to determine if IL-3 was important in iTreg induction in UCB, WCB was cultured with either recombinant IL-3 or with an anti-IL-3 neutralizing antibody. If IL-3 signaling influences iTreg induction, then the addition of IL-3 may increase iTreg frequency, while neutralizing IL-3 will block iTreg differentiation. Cells were cultured for fourteen days and Treg frequency was determined by FACS. The frequency of Tregs is considerably reduced when WCB is treated with anti-IL-3 neutralizing antibody in all of the aforementioned populations seen in the CD4/CD8 profile when compared to untreated WCB (Figure 10B). The addition of IL-3 maintains the Treg frequency in all populations when compared to untreated WCB (Figure 10B); however, there is an increase in the frequency of CD4⁺ cells and a decrease in the frequency of CD8⁺ cells (Figure 10A). Histogram analysis of CD4⁺CD8⁻ cells in untreated WCB, exogenous IL-3 treated WCB, and anti-IL-3 antibody treated WCB displays varying expression of CD25; however, only a decrease in Foxp3 was observed in anti-IL-3 treated WCB (Figure 11). An increase in CD25 expression was observed in WCB treated with exogenous IL-3 while a decrease in CD25 expression was seen in WCB treated with anti-IL-3 antibody when compared to untreated WCB (Figure 11A). These trends were observed in all gated populations (data not shown). These data suggest that IL-3 may be vital for the induction of iTregs in UCB.

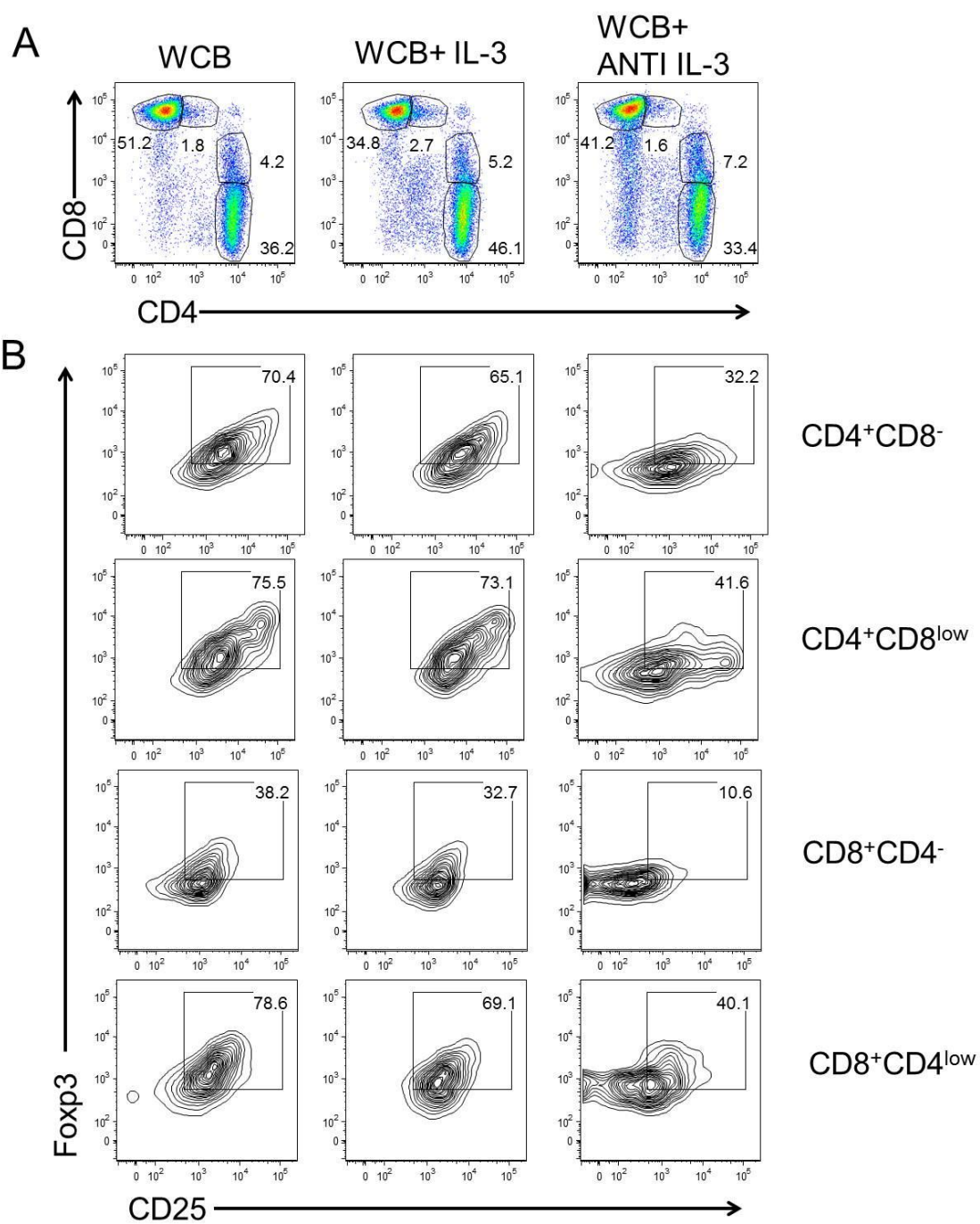


Figure 10: Treg frequency of WCB with exogenous IL-3 or anti-IL-3 treatment.

WCB cultures are stimulated with α -CD3 and IL-2 with or without the addition of either exogenous IL-3 or anti-IL-3 antibody. Cells were harvested at day 14 and stained for CD4, CD8, CD25 and Foxp3. Cells are gated on CD4⁺CD8⁻, CD4⁺CD8^{low}, CD8⁺CD4⁻ and CD8⁺CD4^{low} (A). Treg frequency is determined by CD25 and Foxp3 expression (B).

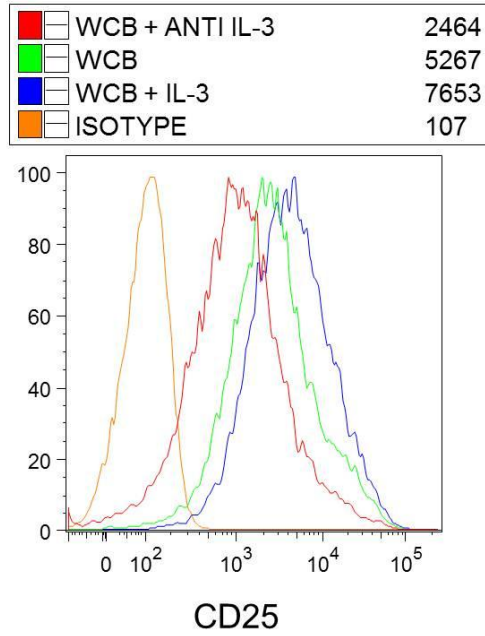
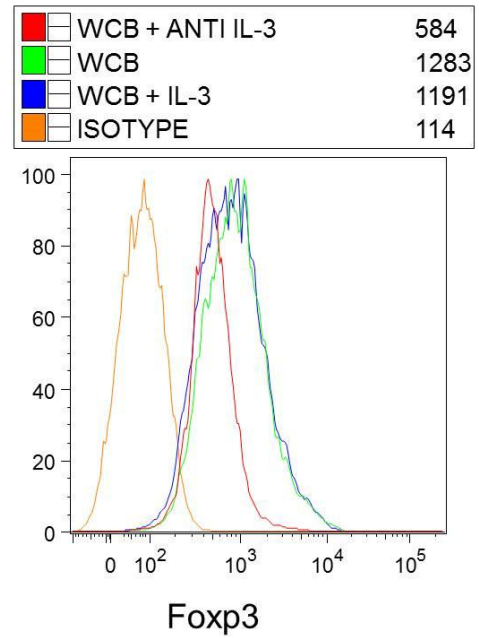
A**B**

Figure 11: Foxp3 and CD25 expression with exogenous IL-3 or anti-IL-3 treatment.

WCB cultures are stimulated with α -CD3 and IL-2 with or without either exogenous IL-3 or anti-IL-3 antibody. Cells were harvested at day 14 and stained for CD4, CD8, and CD25 and Foxp3. Cells are gated on CD4⁺CD8⁻ and CD25 (A) and Foxp3 (B) expression is observed.

CHAPTER FOUR

DISCUSSION

Introduction

Regulatory T (Treg) cells are a subset of T cells that modulate immune responses by maintaining homeostasis and self-tolerance. When an immune response is mounted against self-tissue, autoimmunity ensues; thus, Treg function is critical to negatively regulate any unwanted immunogenic reaction. The absence or dysfunction of Tregs results in a wide array of diseases which includes IPEX, IBD, MS, cancer, allergy and infection. Two subsets of Tregs include nTregs and iTregs and it has been proposed that the mechanism of development is distinct between the subsets (13). Many factors are involved in the differentiation and function of immune cells. Understanding what factors are important for producing inducible Tregs will enhance our knowledge about the mechanisms required for iTreg induction and may lead to the generation of Treg based therapies.

Our laboratory has discovered that human UCB has an increased propensity for the differentiation of iTregs compared to adult PBMCs. Upon stimulation of TCR with α -CD3 and APC co-stimulation of CD4⁺ cells in the presence of IL-2 in WCB, CD4⁺ T cells up-regulate the transcription factor Foxp3 and the IL-2 receptor α chain, CD25. It has been demonstrated that expression of Foxp3 is stable after fourteen days of culture

and this stable expression is associated with suppressive function. When CD4⁺ cells are stimulated with α -CD3 and α -CD28 coated polystyrene beads in the presents of IL-2, iTreg differentiation is not observed and is used as a non-Treg (CD4⁺CD25⁻Foxp3⁻) control. Other groups have reported that histone modifications and cytokine signaling are two very important signals vital for the differentiation and function of Tregs (14,19,21,23,25,29). This led us to hypothesize that epigenetic histone modifications and cytokine signaling are imperative to control the differentiation of iTregs from UCB. This *ex vivo* system allows us to study and better understand the mechanisms that are influencing iTreg differentiation.

In this study, we focused on two different mechanisms that mediate the processes that may be critical for iTreg differentiation in UCB. First, we determined what epigenetic modifications on histone tails are involved in the activation of gene expression in iTregs. We found that the histone modifications H3K4me3 and H3K9ac decreased dramatically in iTreg cells (CD4⁺CD25⁺Foxp3⁺) compared to non-Treg cells (CD4⁺CD25⁻Foxp3⁻). Alternatively, no difference was seen in H3S10ph and H4K8ac when comparing Treg and non-Treg cells. The cytokine TGF- β is involved in provoking the change in H3K4me3 and H3K4ac observed. These data suggest that histone rearrangement is required for iTreg differentiation and may be mediated through TGF- β signaling. Second, we propose that up-regulation of CD8 on CD4⁺ cells as well as up-regulation of CD4 on CD8⁺ cells may represent a more activated phenotype due to the increase in CD25 and Foxp3 expression. Third, we established that CD4⁺ iTregs express

the IL-3 receptor α chain (CD123) suggesting that IL-3 may be required for iTreg induction in UCB. Addition of anti-IL-3 neutralizing antibody to WCB blocks the biological function of IL-3 and led to the dramatic decrease in expression of iTreg markers CD25 and Foxp3. Conversely, addition of exogenous IL-3 increased expression of CD25 while Foxp3 expression was unchanged compared to untreated WCB. These data propose that IL-3 may be required for iTreg differentiation.

Histone modifications in iTreg cells

Our data reveals that epigenetic modifications of histones are important for the differentiation of iTreg cells (CD4⁺CD25⁺Foxp3⁺) from UCB. The histone modification H3K4me3 and H3K9ac are decreased in iTreg cells while H3S10ph and H4K8ac are unchanged compared to non-Treg cells (CD4⁺CD25⁻Foxp3⁻). To determine if the variation detected was dependent on TGF- β signaling, we added exogenous TGF- β to CD4⁺ bead simulated cultures and TGF- β inhibitor SB-431542 or anti-TGF- β antibody to WCB cultures. The addition of TGF- β to CD4⁺ bead stimulated cells was able to restore expression of CD25 and Foxp3 as well as establish the decrease in H3K4me and H3K9ac seen in iTregs from WCB. Inhibition of TGF- β signaling with the small kinase inhibitor SB-431542 resulted in a decline of CD25 and Foxp3 expression whereas the anti-TGF- β antibody did not have a drastic decrease in CD25 and Foxp3 expression. This may be due to the presents of other molecules that are a part of the TGF- β superfamily such as activin which may be activating the signaling pathway while SB-431542 inhibited kinase activity which completely blocked all signaling. Surprisingly, H3K4me3 and H3K9ac

were unchanged in CD4⁺ cells where TGF- β signaling was inhibited compared to untreated WCB. However, an increase in frequency of CD4^{low} cells was observed and TGF- β inhibition increased H3K4me3 and H3K9ac in CD4⁻ and CD4^{low} cells. These data demonstrate that epigenetic modifications of histones are important for iTreg differentiation and TGF- β signaling may be modulating this chromatin rearrangement.

As previously mentioned, it has been established that TGF- β signaling is an essential factor in T cell homeostasis through many cells of the immune system (20). TGF- β has been shown to be required for the differentiation of iTregs from naïve CD4⁺ T cells in the periphery (22). Activated TGF- β binds to the TGF- β receptor II and then forms a heterodimer with TGF- β receptor I; thus, activating Smad2/3 signaling. Li et al. has shown that mice lacking the TGF- β receptor II result in lethal inflammation (19). The expression of Foxp3 is mediated by TGF- β signaling through the activation of Smad3 and its association with the *foxp3* promoter (23). Ross et al. has shown that Smad2 recruits the histone acetyltransferase p300 to acetylate lysine residue 9 on histone protein H3 to promote active gene transcription and many other histone co-activators have been implicated in Smad2/3 signaling (21). It has also been established that pro-transcriptional modifications H3K9ac at the *foxp3* locus has a strongly associated with Treg cells (14). Our data illustrate that TGF- β is mandatory for modulating chromatin rearrangement by H3K4me3 and H3K9ac histone modifications. Overall, we propose a model that after stimulation with TGF- β , naïve CD4⁺ T cells increase the expression of Foxp3 and CD25 through TGF- β signaling by first inducing changes in epigenetic modifications of amino

acid residues at histone tails which subsequently allows binding of Smads to the open promoter of genes important to iTreg differentiation and function.

CD4, CD8 and CD123 expression on iTregs

As described above, TGF- β inhibition resulted in an increased frequency of CD4^{low} cells. Monocytes express CD4 at low levels and can differentiate into various APC subsets including plasmacytoid DCs (pDC) (33). An identifying marker for pDCs is the IL-3 receptor α chain, CD123, and it has been shown that pDCs are capable of inducing Treg differentiation resulting in suppressive function (34). We wanted to determine if the CD4^{low} cells were iTregs or monocytes differentiating into pDC's. To address this question we used FACS cell surface staining to phenotype the CD4^{low} cells. These CD4^{low} cells expressed the T cell markers CD8 and CD3 after fourteen days of culture. Four populations were seen when looking at the CD4 and CD8 profile. A population CD4⁺ cells had CD8 up-regulated on the cell surface and another population of CD8⁺ cells had CD4 up-regulated on the cell surface. Sullivan et al. has demonstrated that increased expression of CD4 on CD8⁺ cells is an additional marker for activated CD8 T cells as these CD8⁺CD4^{low} cells expressed higher levels of the activation markers, CD95, CD28, and CD25 when compared to CD8⁺CD4⁻ cells (35). Foxp3 and CD25 expression was increased in the CD4⁺CD8^{low} and CD8⁺CD4^{low} cells when compared to CD4⁺CD8⁻ and CD8⁺CD4⁻ respectively. CD8⁺ cells have been shown to up-regulate Foxp3 and have been implicated in GVHD (8). Thus, the up-regulation of CD4 on CD8⁺ cells and CD8 on CD4⁺ cells seen in our UCB system may constitute an additional

marker for a more activated iTreg; however, iTreg induction in CD8⁺ cells has not been well characterized in this UCB system. Surprisingly, we observed that CD4⁺CD8⁻ and CD4⁺CD8^{low} cells expressed high levels of CD123 while CD8⁺CD4⁻ and CD8⁺CD4^{low} cells did not, suggesting that IL-3 signaling may be important for CD4⁺ iTreg induction.

IL-3 signaling and iTreg differentiation

We have demonstrated that IL-3 receptor α chain, CD123, is expressed on CD4⁺ iTregs after fourteen days of culture. Hence, we determined if IL-3 signaling is important for the differentiation of CD4⁺ iTregs from UCB. We added anti-IL-3 antibody to neutralize the biological function of any IL-3 present in culture or exogenous IL-3 to WCB. Our data show that when IL-3 is neutralized, there is a decrease in the expression of CD25 and Foxp3 in all four populations observed in the CD4/CD8 profile. We observed an increased frequency of CD4⁺ cells and a decreased frequency of CD8⁺ cells when WCB was treated with exogenous IL-3. The addition of exogenous IL-3 had no effect on Foxp3 expression; however, there was an increase in CD25 expression in all of the aforementioned populations. These data suggest that IL-3 signaling is required for iTreg differentiation.

Interleukin 3 signaling is mediated through signal transducers known as STATs. IL-3 binds to its cognate receptor, CD123, which allows the association of the IL-3 common β chain (IL-3 β_c); thus, leading to activation of a tyrosine protein kinase, JAK2. This subsequently activates STATs, specifically STAT5, which forms a dimer to enter the nucleus and modulates gene expression. IL-3 has a wide range of functions and it has

been previously reported that IL-3 is produced by activated T cells; thus, I hypothesize that IL-3 is being produced by activated T cells in our UCB model. Mirabella et al has shown that during T cell development, the *IL3* locus goes through progressive stages of development (37). Immature CD4⁺CD8⁺ double positive thymocytes have the *IL3* gene epigenetically silent in order to stop any inappropriate cell activation during TCR selection (37). Naïve CD4⁺ T cells show low levels of IL-3 expression; however, after TCR stimulation, the *IL3* locus undergoes chromatin rearrangement allowing for intermediate transcription of the *IL3* gene (37). Furthermore, the epigenetic imprint is maintained in memory T cells which expresses 4 fold more IL-3 mRNA compared to naïve cells (37). It is also interesting to note that Th2 cells contain higher levels of IL-3 mRNA when compared to Th1 cells (37). Therefore, IL-3 produced by activated T cells in UCB may activate the CD14⁺CD36⁺ monocytes that express CD123 at day zero to boost their ability to induce iTreg differentiation. Additionally, when CD4⁺ cells up-regulate CD123 on the cell surface upon TCR activation, IL-3 may be working in an autocrine and/or paracrine manner to promote the induction of iTreg cells.

Seivastava et al. has demonstrated that IL-3 enhances the frequency of Foxp3⁺ T cells that are able to suppress the function of effector T cells (25). Furthermore, STAT5 knockout mice have decreased Treg number and defective function of Tregs (29). Nakajima et al demonstrates that STAT5^{-/-} splenocytes have reduced CD25 mRNA compared to wild type, suggesting that STAT5 is required for CD25 expression which is required for iTreg proliferation (30). These data suggest that IL-3 is important for iTreg differentiation and we propose IL-3 is important for modulation of CD25 and Foxp3

expression. In the future, it would be interesting to further characterize the role of IL-3 signaling in iTreg differentiation as its importance has been only recently uncovered.

Global Signaling

We have demonstrated that epigenetic histone modifications, TGF- β , and IL-3 may all be important for the differentiation of iTregs from UCB. A cell receives a wide array of signals which must jointly operate to change the gene expression pattern that is required for any cell to differentiate. For the induction of iTregs from UCB, it is not merely one signal that instructs a naïve CD4⁺ T cell to express proteins important for iTregs such as Foxp3 and CD25; thus, a cascade of events must take place for a cell to implement the gene expression pattern important for iTregs differentiation and function.

Epigenetic histone modifications are not dependent on the sequence of DNA, and therefore, a signal must be received in order to induce these changes. DNA de-methylation has been associated with histone acetylation and active transcription (14). Importantly, DNA methylation status is evolutionarily conserved at specific regions (14). Other signals that may promote the rearrangement of chromatin include TGF- β signaling by recruiting histone modifying enzymes such as histone acetyltransferases to further induce epigenetic changes at promoter regions that support or suppress transcription (21). Ross et al. has demonstrated that Smad2/3, the signal transducer for TGF- β signaling, associates with the acetyltransferase p300 which is involved in acetylation of histone tails (21). Furthermore, Tone et al. also shows the importance of Smad3 for histone modifications and then binding to an enhancer region of the *foxp3* promoter once the

binding site is accessible; however, Smad3 is not mandatory for maintained expression (23). TGF- β signaling is involved in the differentiation of many other T cell subsets such as Th9 and Th17; consequently, additional factors are a necessity for specific up-regulation of genes important for iTreg differentiation.

Once the chromatin has been remodeled and is in an open confirmation, other transcription factors such as STATs can bind for sustained transcription of genes. Passerini et al. reveals that cytokines involved in STAT5 signaling are required for Foxp3 expression in CD4⁺CD25⁺ Tregs (38). Furthermore, Yao et al. has demonstrated that there are STAT5 binding sites in highly conserved regions of the *foxp3* promoter (39). Thus, I hypothesize that TGF- β signaling first induces changes in histone modifications through the association of Smads with histone modifying enzymes (Figure 12). This is only a preliminary step towards iTreg differentiation. Once the chromatin is in an open confirmation, Smads are capable of binding to the open promoter and may serve as an additional signal to promote binding of other factors to support transcription of the gene. This however is not specific for iTreg differentiation as TGF- β is involved in the induction of other cells types. Another molecule that may be specific for iTregs differentiation may include IL-3. The signaling pathway of IL-3 may influence CD25 and Foxp3 transcription through STAT5 binding to the promoter region to provide stable expression (Figure 12). Therefore, all of these factors as well as RNA polymerase may form a complex in order to stabilize the transcription of genes required for iTreg induction.

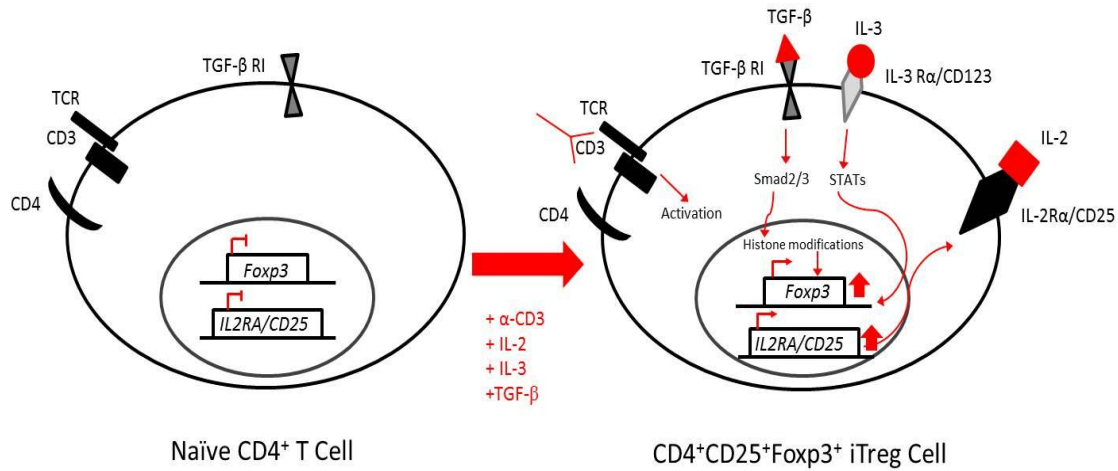


Figure 12: Mechanistic hypothesis. In WCB cultures stimulated with α -CD3, exogenous IL-2, exogenous IL-3 and TGF- β , naïve CD4⁺ T cells can send a signaling cascade through the cell to regulate histone modifications as well as genes expression for proteins that are important for Treg function such as Foxp3 and CD25.

Concluding remarks

My work has exposed important mechanisms that are required for the differentiation of iTregs from human UCB. My data suggest that epigenetic modifications of amino acid residues at histone tails are imperative for the expression of genes required for Treg function. In addition, TGF- β signaling may be important in modulating these epigenetic changes through Smad2/3 association with histone modifying enzymes. Also, it is important to note that expression of low levels of CD4 on CD8⁺ T cells and low levels of CD8 on CD4⁺ T cells correlate to an increase in CD25 and Foxp3 suggesting up-regulation of these surface markers denote an additional phenotype of iTregs.

Additionally, a novel mechanism that may be crucial for iTreg differentiation may be through IL-3 signaling. IL-3 signaling may be required for maintained CD25 and Foxp3 expression through STAT5 association with the promoter region of these genes. Both aforementioned signals must work in an organized fashion for the differentiation of iTregs; furthermore, appreciating the mechanisms required for iTreg induction may lead to the production of Treg therapeutics to treat immune dysfunction.

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VITA

The author, Rebecca Krier, was born in Arlington Heights, IL on July, 26th 1987 along with her identical twin sister Elizabeth to James and Karen Krier. She received a Bachelor of Science in Molecular and Cellular Biology with a minor in Chemistry from University of Illinois at Urbana-Champaign. After graduation, Rebecca worked as a research assistant in the lab of Dr. Rong Yuan, The Jackson Laboratory, Bar Harbor, Maine (January 2010- June 2011).

In August of 2011, Rebecca enrolled in the graduate program at the Infectious Disease and Immunology Institute, Loyola University Chicago and joined the lab of Dr. Makio Iwashima where she studied inducible regulatory T cells in an umbilical cord blood model.